Ultrasonographic Development of the Fetal Sheep Stomach and Evaluation of Early Gestation Ultrasound-guided *In Utero* Intragastric Injection

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SUMMARY

Objective: Safely targeting the fetal gastrointestinal tract during early gestation is essential to develop effective prenatal gene therapy for gastrointestinal diseases. In this study, we aimed to characterize the development of the fetal sheep stomach sonographically and to determine the optimum gestational age, as well as the short-term morbidity and mortality of early-gestation ultrasound-guided intragastric injection.

Materials and Methods: In experiments investigating ultrasound-guided prenatal gene therapy, we studied the size and development of the stomach of 185 sheep fetuses (33–144 days' gestational age [GA]; term is 145 days). Ultrasound-guided intragastric injection was performed in 12 fetuses at 55–62 days' GA and postmortem examinations were performed 48 hours later.

Results: The stomach was not visible at or before 40 days' GA, but it was seen in all fetuses at 55 days' GA or more. The anteroposterior, transverse and longitudinal diameters of the stomach increased in a quasi-linear fashion throughout gestation. Intragastric injection was successful in 10 out of the 11 fetuses (91%) injected at 60–62 days' GA, with nine fetuses (91%) surviving this procedure.

Conclusion: In the early-gestation sheep fetus, ultrasound-guided intragastric injection has a good success rate with a low short-term mortality and morbidity. [*Taiwan J Obstet Gynecol* 2010;49(1):23–29]

Key Words: fetus, gene therapy, injection, sheep, stomach, ultrasound

Introduction

The fetus is increasingly being seen as a target for prenatal treatments such as stem cell transplantation and gene therapy. At present, there are few indications to access the fetal gastrointestinal tract for diagnosis or therapy. However, genetic conditions such as cystic fibrosis (CF) and congenital enteropathies may be amenable to prenatal cell or gene-based treatments through minimally invasive techniques that could be clinically applied during the early-gestation fetal immunologic window [1].

The target organ for treatment of CF and congenital enteropathies is the small bowel. In 60% of CF fetuses, thickened intestinal mucus and undigested proteins block the small bowel, causing meconium ileus, a life-threatening complication [2]. Adult CF patients also suffer from recurrent episodes of pain, abdominal

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distension and bilious vomiting, called distal intestinal obstruction syndrome [3]. However, delivering therapy directly into the small bowel is difficult to achieve owing to its small size. In the human fetus, for example, tiny areas of fluid can be seen within the small bowel lumen from 13 weeks' gestational age (GA), but ultrasound-guided injection would not be possible until 25 weeks' GA, when the average diameter of the small bowel lumen is 1.8 mm [4].

In sheep, as in all ruminants, the stomach is highly specialized. It is composed of four chambers, the rumen, reticulum, omasum and abomasum, through which food passes successively during rumination (Figure 1). Little is known about the ultrasonographic dimensions and morphology of the stomach in the sheep fetus. It has been suggested that the stomach in fetal sheep becomes visible under ultrasound somewhere between 40 and 65 days' GA [5]; however, an exact time frame and related stomach dimensions are not available. We studied the sonographic visibility of the fetal sheep stomach throughout gestation and evaluated the intragastric injection technique in early-gestation sheep fetuses to determine the optimum time frame, the morbidity, and the mortality of the procedure.

Materials and Methods

Biometry and morphology of the ovine fetal stomach

During experiments designed to investigate ultrasound-guided prenatal gene therapy [6–11], 185 Romney sheep fetuses underwent 238 ultrasonographic examinations between 33 and 144 days' GA (term is 145 days). Each fetus was examined a maximum of two times. Ewes were time-mated after receiving intravaginal progesterone suppositories for 2 weeks to induce ovulation. General anesthesia was induced with 1 g of thiopentone intravenously, and after intubation, the ewes were maintained on halothane 2% in oxygen. Transabdominal ultrasound imaging was performed using a 3.5 MHz probe on a Vingmed Sonotron CFM 800 (GE Vingmed UltraSound, Horten, Norway) or an Acuson 128 XP10 ultrasound scanner (Siemens, Bracknell, UK). Fetal biometry (biparietal diameter, occipito-snout length, abdominal circumference, and femur length) was performed to confirm GA according to published normal range measurements [12,13].

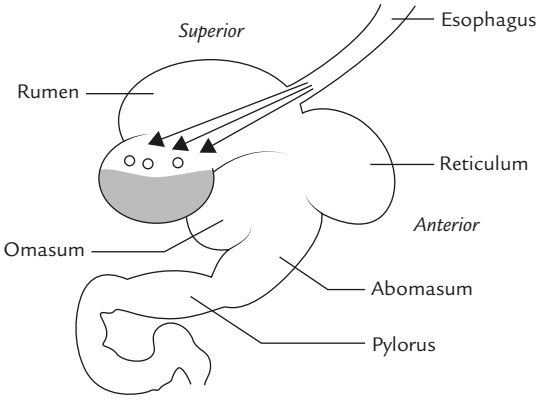

Figure 1. Chambers in a sheep's stomach.

The fetal stomach chambers were visualized in the upper left abdomen, and the number of stomach cavities was documented. The widest anteroposterior (AP) and transverse (T) diameters were measured in the axial plane, and the widest longitudinal (L) diameter was measured in the sagittal plane. Each diameter measured included all the communicating stomach chambers that were visible in that plane. In all measurements, the calipers were placed "inner-to-inner". The presence of a normally developed stomach was confirmed at post-mortem analysis in all fetuses.

Injection of the fetal sheep stomach

Twelve Romney sheep fetuses between 55–62 days' GA were used in these experiments. Under general anesthesia and after ultrasound measurement of the fetal stomach, a 22-gauge Echotip spinal needle (Cook Ireland Ltd, Limerick, Ireland) was inserted under ultrasound guidance through the maternal skin, the uterus and into the fetal stomach, avoiding passage through the placentomes if possible. Correct needle placement was confirmed by aspiration of 100 μL of gastric fluid after which, adenovirus vectors containing the β-galactosidase reporter gene (adenovirus-lacZ) [14] and transduction enhancing agents [11] were injected under view. The presence of microbubbles within the stomach cavities was used to confirm correct fluid placement. The needle was then flushed through with 40 μL saline to clear the needle dead space. The time taken to inject the stomach cavity was measured from the first insertion of the needle through the fetal skin to removal of the needle from the fetus after successful delivery of vector and transduction enhancing agents. The ewes were then allowed to recover after extubation. Following the injection, fetal survival was monitored every 12 hours for 2 days using ultrasound while the ewe was awake. Two days after injection, the ewes were euthanized using an overdose of intravenous pentobarbital sodium (Euthatal; Rhône Merieux, Essex, UK), a post-mortem examination was performed and tissue samples were processed as described previously [6]. All procedures and ultrasound examinations were conducted in accordance with UK Home Office regulations and the Guidance for the Operation of Animals (Scientific Procedures) Act (1986).

Results

Measurement of the fetal sheep stomach

The dimensions of the fetal stomach and the number of visible chambers were documented in 190 and 211 examinations, respectively. The fetal stomach was not visible in any fetus aged 40 days' GA or less ($n = 14$). In three fetuses aged between 45 and 50 days' GA, the fetal stomach was visible; but because of the small numbers, no conclusions can be drawn. As of 50 days' GA, more data were available. Between 51 and 54 days' GA, the fetal stomach was visible in 43 out of 55 fetuses (78%) as compared with 100% when the fetuses were examined at or beyond 55 days' GA (χ^2 test, p value < 0.001).

The ability to visualize the stomach chambers was studied throughout gestation. As gestation advanced, more of the stomach chambers became visible (Table 1). The fetal stomach dimensions increased in a quasi-linear fashion from 45 to 144 days' GA; the largest diameter at any time point was the longitudinal diameter. Figure 2 shows the change in dimension of the AP, T and L diameters of the stomach from 45 to 144 days' GA.

Ultrasound-guided intragastric injection

We found injection at 55 days' GA difficult owing to the small cavity volume at this age (2.7 mm AP × 3 mm T × 5 mm L). In an attempt to inject the fetal sheep stomach at this GA, the dimensions did not allow withdrawal of gastric fluid into the syringe. Therefore, correct needle placement could not be confirmed. A 22-gauge needle tip was successfully advanced into the fetal stomach, allowing fluid turbulence to be visualized, and microbubbles were seen within the stomach lumen on instillation of 100 μL saline and the viral vector. Fetal demise was noted 12 hours after injection when postmortem examination showed a large peritoneal blood clot anterior to the liver and umbilical vein, and a smaller clot to the right of the fetal rumen, suggesting iatrogenic death. Bacteriologic culture of fetal tissues showed a moderate growth of *Campylobacter jejuni*, a fleece commensal.

Because of the difficulty involved in injecting the fetal stomach at 55 days' GA and our observation that the AP diameter was approaching 10 mm at 60 days' GA, we performed further stomach injections from this GA onwards. Injection was attempted in 11 fetuses from seven ewes at 60–62 days' GA with success in 10 fetuses (91%). In all cases, the needle was confirmed to be correctly positioned within the fetal stomach by withdrawal of 100 μL of clear gastric fluid. In some of the fetuses, we were able to demonstrate all four stomach chambers after injection of an additional volume of fluid (300–1,000 μL), compared with only two prior to injection, clearly demonstrating that the injected fluid passed into the fetal stomach chambers distal to the injection site (Figure 3). Evidence for definitive gene transfer to the distal gastrointestinal tract in the surviving successfully injected fetuses has been already presented elsewhere [15]. One injection failure was in a triplet pregnancy, in which the other two fetuses were successfully injected. Visualization of the third triplet was poor owing to fetal position, and the injection was abandoned after failure of attempts to withdraw gastric fluid.

In most cases (eight out of 11 injections, 73%), the fetus was positioned so that the needle transgressed the abdominal cavity a short distance before entering the stomach. In three cases, the fetus was lying on its left side, meaning that to reach the stomach, the needle had to pass through the fetal liver. These cases were not associated with failed injection, hemorrhage or infection. Overall, the mean time to successful gastric injection was 7 minutes, 21 seconds (standard deviation, 6 minutes 44 seconds; range, 29 seconds to 13 minutes 34 seconds). In one twin fetal pair, the first attempt at gastric injection failed owing to fetal position, but a second attempt was successful in each case. Intragastric injection was achieved at the first attempt

Table 1. Development and visibility of the fetal sheep stomach chambers through gestation

GA range (d)	*n*	Stomach chambers visible, *n* (%)				
		0	1	2	3	4
33–40	14	14 (100)	0	0	0	0
45–50	3	0	3 (100)	0	0	0
51–54	54	12 (22)	27 (50)	14 (26)	0	1 (2)
55–64	63	0	24 (38)	38 (60)	1 (2)	0
65–80	22	0	3 (14)	16 (72)	3 (14)	0
81–100	28	0	0	30 (11)	9 (32)	16 (57)
101–115	17	0	0	5 (29)	3 (18)	9 (53)
116–144	10	0	0	0	0	10 (100)

GA = gestational age.

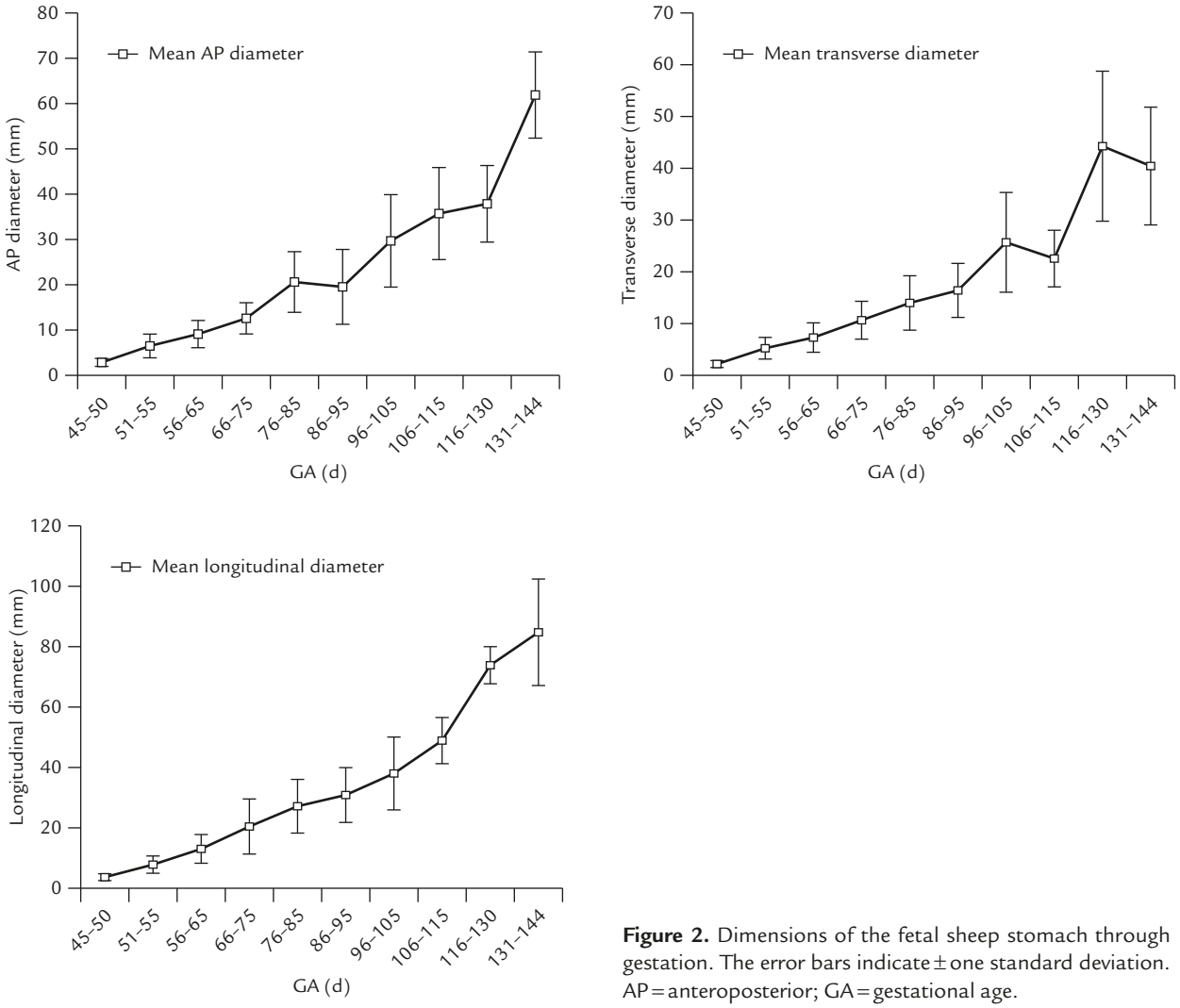

Figure 2. Dimensions of the fetal sheep stomach through gestation. The error bars indicate ± one standard deviation. AP = anteroposterior; GA = gestational age.

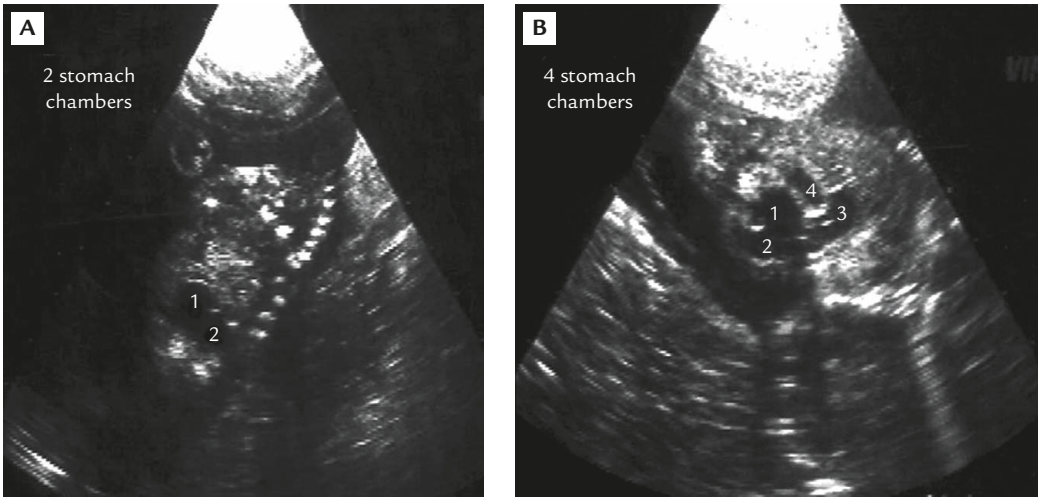

Figure 3. Sonograms showing injection of fluid into the stomach of a fetal sheep at 60 days' gestation. There was an increase in the number of stomach chambers visible from (A) 2 days before injection to (B) 4 days after injection.

in eight fetuses (80% of successful injections) in a mean time of 4 minutes 56 seconds (standard deviation, 2 minutes 41 seconds; range, 29 seconds to 8 minutes 18 seconds).

There was one fetal death in the 11 injected fetuses at 60–62 days' GA (91% survival). This occurred in a twin gestation shortly after injection, in an experiment in which both twins received gastric injection. Postmortem

Table 2. Postmortem and histologic findings after ultrasound-guided intragastric injection of the fetal sheep stomach

Age (d)	Vector and enhancing agents				Postmortem examination findings	Ascites (mL)	Histologic and culture examination
	AdlacZ (p/kg)	DEAE-dextran (5 mg/mL)	Sodium caprate 100 mM (mL)	Perflubron (mL)			
62	1×10^{10}	–	100	–	No abnormality	1	No abnormality
60	1.6×10^{13}	–	–	–	No abnormality	2	No abnormality
61	1×10^{13}	–	100	0.3	No abnormality	2	No abnormality
60	9.5×10^{12}	+	100	0.3	No abnormality	2	No abnormality
60	1.4×10^{13}	+	200	1.0	No abnormality	0.5	No abnormality
60	1.0×10^{13}	+	200	1.0	Fine peritoneal adhesions	1	Coagulase-negative *Staphylococcus* cultured from ascites
60	1.1×10^{13}	+	200	1.0	No abnormality	6.5	No abnormality
60	1.1×10^{13}	+	200	1.0	Fetal death; peritoneal clot	–	Tissue autolysis, no bacteria cultured
60	1.0×10^{13}	+	200	1.5	No abnormality	3	No abnormality
60	8.8×10^{12}	+	200	1.5	Small pleural effusion	8	No abnormality
55	2×10^{11}	–	–	–	Fetal death; peritoneal clots	–	*Campylobacter jejuni* cultured in some fetal tissues
60	Failed intragastric injection				No abnormality	0.1	No abnormality

AdlacZ = adenovirus-lacZ; DEAE = diethylaminoethyl.

analysis showed a large blood clot posterior to the right lobe of the liver, extending to the left of the midline. Culture of fetal tissues showed no evidence of bacterial infection, and we concluded that the cause of death was procedural rather than owing to iatrogenic infection.

Postmortem and histologic findings 2 days after vector injection are shown in Table 2. Clear ascitic fluid was present in all nine fetuses who survived, and at a larger volume than is normally present in the omentum at this GA (50–100 μL). Measurement of the protein content in four of the ascitic fluid samples confirmed a transudate. The volume observed was generally proportional to the volume of fluid injected into the fetal stomach cavity, and it is likely that this was fluid that had leaked out of the stomach after injection. In the fetus with the largest volume of ascites (8 mL), a small pleural effusion (0.2 mL) was also observed. In another fetus, filmy peritoneal adhesions were seen. Culture of the fetal liver and ascites showed a pure growth of coagulase-negative *Staphylococcus* that was most likely introduced during the injection procedure.

Discussion

As a first step towards clinical application of prenatal gene therapy for CF, we have characterized the ultrasonographic dimensions and morphology of the ovine fetal stomach throughout gestation and evaluated a minimally-invasive ultrasound-guided injection technique into the fetal stomach in a large animal model in early gestation. We found that the fetal sheep stomach could be reliably observed by ultrasound from 55 days' GA. This is comparable to human pregnancy in which the stomach could be seen using transabdominal ultrasound in 98% of fetuses from 14 weeks' GA; indeed, an inability to visualize the stomach at this age was associated with an abnormal pregnancy outcome [16]. Transvaginal sonography in the human fetus permits earlier visualization of the stomach from 9 weeks, with the stomach being visible in almost all cases at 12 weeks' GA. This is equivalent to 46 days' GA in the fetal sheep [17]. Transvaginal sonography has not been used in fetal sheep, but it is likely that fetal anatomy would be visible earlier in gestation using this method. Our study would suggest, however, that even if the stomach were visible, attempts to inject it might fail because of the small size.

The widest diameters of the fetal sheep stomach showed an almost linear increase during gestation. We did not calculate the total volume of the fetal sheep stomach throughout gestation, however, because of its more complicated anatomy and design for rumination in the sheep. In the human fetus, there is a wide variation

in stomach volume because of dynamic filling and emptying [18]; but over long periods of evaluation, the dimensions remain relatively constant [19]. Using three-dimensional reconstruction from two-dimensional ultrasound measurement, the stomach volume has been shown to increase linearly in the latter half of pregnancy [20]. The four chambers of the sheep stomach develop at different rates over the GA range we studied. From 18 days' GA, the primitive stomach is identifiable as an expansion of the foregut spindle in the early sheep embryo, and the four chambers are discernible anatomically by 26 days' GA [21]. By 34 days' GA, the rumen and reticulum are clearly separated, with the rumen on the left side of the abdominal cavity. Rapid early expansion of the rumen pushes the omasum and abomasum to the right of the abdominal cavity, and by the end of the embryonic period, the position and relative sizes of the chambers are the same as in the adult with the rumen comprising around 75% of the stomach volume. In the second half of gestation, the abomasum expands preferentially, so that at birth, it accounts for more than half of stomach volume. This is because fermentation is unnecessary in the suckling neonate. Ruminal growth only catches up after birth on weaning and the relative sizes of the chambers reach that of the adult again by 1 year of age. It is most likely that the one or two chambers visible sonographically in early gestation represented the reticulorumen, and the enlarging abomasum and omasum most probably were the third and fourth chambers seen later in gestation or after gastric distension that are noted caudal and to the right of the reticulorumen on longitudinal views of the fetus (Figure 3B).

We found that intragastric injection was best achieved from 60 days' GA, when the AP diameter was approaching an average of 10 mm. This is still below 70–72 of 145 days' GA, after which the fetal sheep immune system becomes significantly less tolerant to a foreign antigen [1]. Intragastric injection was achieved in all but one case complicated by a triplet pregnancy and in a relatively short procedure time. An advantage of the technique is that the needle position could be confirmed by withdrawal of a small volume of gastric fluid. The needle position was also confirmed when needle advancement within the cavity was associated with fluid turbulence, and microbubbles were clearly seen within the stomach lumen during fluid instillation. The appearance of additional stomach chambers during injection is another sign of a successful injection; however, this sign was not consistently observed in all of the successfully injected cases.

The short-term morbidity and mortality from the procedure were low. The position of the fetus did not appear to affect the outcome of the procedure, but larger numbers of injections are needed to confirm this finding. In a clinical setting, the risk of trauma to intraperitoneal organs might be reduced by injecting the fetal stomach only when it is positioned uppermost and closest to the uterine wall. It is likely that procedures would be done without maternal or fetal sedation, and therefore, the injection could be performed when the fetus had moved into the optimum position. We performed postmortem and histologic analysis 2 days after injection, but longer-term follow up, including allowing some lambs to deliver, is necessary to evaluate the effect of intragastric injection on the development of the fetal stomach and its function in postnatal life. As anticipated, we observed hemorrhagic and infective complications. Peritonitis and intraperitoneal adhesions are likely to be the most common complications.

There is one published study of ultrasound-guided intragastric injection of late-gestation fetal rabbits [22]. The authors successfully injected the stomach in 70% (18/26) of cases. This was lower than our success rate of 91% (10 out of 11 cases) and may be because of the double needle technique that the authors used in which they placed a 20-gauge spinal needle into the fetal abdominal cavity and then advanced a 26-gauge needle through this into the fetal stomach. Their short-term survival rate (91%) was good, and there was no histologic evidence of trauma or hemorrhage in the survivors.

In summary, we have characterized the ultrasonographic appearance and dimensions of the fetal sheep stomach during gestation and have shown that the fetal sheep stomach can be reached by ultrasound-guided injection in the early gestation, pre-immune fetus with minimal trauma and low short-term mortality. Our findings, combined with effectiveness in achieving a widespread fetal gene transfer to the gastrointestinal tract and a similar ontogeny of human and ovine fetal gut peristalsis and gastric acid secretion [15], suggest that clinically applicable studies in the fetal sheep are appropriate for the development of prenatal gene therapy of congenital diseases that affect the gastrointestinal tract. Despite differences in stomach anatomy, the shape and size of the fetal sheep rumen in early gestation is very similar to that of the stomach in the human fetus [19]. We anticipate, therefore, that injection of the stomach in the human fetus would be just as straightforward.

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